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Hetero-bivalent Imaging Agents for Simultaneous Targeting Prostate-Specific Membrane Antigen (PSMA) and Hepsin

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14. ABSTRACT

The hypothesis of the original proposal was to discover novel imaging probes for the diagnosis of prostate cancer by targeting simultaneously PSMA and hepsin, which are highly expressed in advanced and metastatic prostate cancer. In Year 3, we successfully established the synthetic strategy for PSMA-hepsin heterobivalent ligands and synthesized two dual-targeting conjugates **12**-**13** labeled with optical dye Cy 5.5 and Cy 7, respectively. We also synthesized two peptide-based IPLLVVPL analogs (**15**,**17**) linked with DOTA and evaluated their inhibitory activities against hepsin protease. With respect to biological experiments, we developed the PC3/ML cell lines which express PSMA, hepsin, and PSMA/hepsin for *in vitro* cell uptake and *in vivo* imaging studies. Compound **13** showed a low but detectable increased cell uptake into the developed cell lines as compared to the control. From these results, we will carry out structural optimization of compound **13** and evaluate their biological activities *in vitro* and *in vivo* in Final Year.

15. SUBJECT TERMS
PSMA, Hepsin, Prostate Cancer, Molecular Imaging

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A. Introduction

Prostate cancer (PCa) is the leading cancer in the U.S. population and the second leading cause of cancer death in men. (1) An estimated 238,590 new cases and 29,720 deaths of prostate cancer will occur in the US during 2013, according to the Cancer Facts & Figures 2013 published by American Cancer Society (www.cancer.org). Even if current prostate cancer screening with the prostate-specific antigen (PSA) blood test have advanced significantly for the early diagnosis of patients with PCa, the controversy on the efficacy of PSA testing for reducing PCa deaths is currently still being debated. There have been comprehensive clinical studies whether PSA testing is an efficient biomarker in diagnosing PCa and reducing PCa deaths. Two European studies reported that men who receiving PSA screening can lower the risk of deaths from PCa while the study in US did not show any difference between PSA screening group and non-PSA group statistically. (2-4) Due to the lack of PSA specificity for PCa, there has been an increase of unnecessary biopsies/treatments of what would be benign or indolent disease state. Therefore, there is an urgent need to explore biomarkers for early and precise detection of PCa, in particular small lesions, i.e., recurrent tumors in the surgical bed, local lymph node invasion and other subtle manifestations of the disease in men. Prostate-specific membrane antigen (PSMA) and hepsin are on the line as clinical biologic markers of PCa due to the fact that they are highly expressed in advanced and metastatic prostate cancer.

PSMA is a type II integral membrane metalloprotease that has abundant and restricted expression on the surface of prostate carcinomas, particularly in androgen-independent, advanced and metastatic disease. (5,6) PSMA possesses the criteria of an ideal target for immunotherapy and diagnosis, i.e., expression primarily restricted to the prostate, abundantly expressed as protein at all stages of the disease, presented at the cell surface but not shed into the circulation, and association with enzymatic or signaling activity. (6) SPECT-CT scan of PCa using ^{111}In-capromab pendetide (Cyt-356, ProstaScint), an [^{111}In]-labeled monoclonal antibody to PSMA, showed promise in the clinic for identifying metastatic tumors in lymph node. (7, 8)

Because of the important functions of PSMA for PCa, there have been a lot of reports on PSMA-based imaging probes which were evaluated *in vivo* by means of PET, SPECT, and optical imaging techniques. (9) According to the PSMA crystal structures, the PSMA active site consists of two distinct subpockets, which form a 'glutamate-sensor' S1' site (pharmacophore) and an amphiphilic S1 site (non-pharmacophore). The cylinder-shape ~20Å deep tunnel region exists adjacent to the S1 site and projects toward the hydrophilic surface of the enzyme. (10) The structural freedom by the S1 site provided diverse modification of PSMA-based imaging probes. In particular, the lysine in the P1 site was utilized as a key scaffold in order to 1) take advantage of the many radiohalogenation methods and radiohalogenated prosthetic groups developed previously for reacting with the ε-amino group of lysine residues, and 2) increase the structural diversity of urea-based PSMA inhibitors. (10, 11, 12)

Hepsin is a type II transmembrane serine protease which is preferentially expressed in neoplastic prostate over benign prostate. (13) In addition, the mRNA level of *hepsin* was elevated in ~ 90% of PCa specimens and was > 10-fold higher in metastatic PCa than in normal prostate or benign prostatic hyperplasia (BPH). Hepsin is composed of 413 amino acids and a large C-terminal residue is located in the extracellular region. The serine protease domain at the C-terminus extracellular part is highly homologous among type II trypsin-like serine proteases.(14) There have been no reports on hepsin inhibitors that have a nanomolar IC_{50} value, which is considered as a cut-off criterion to assess potential candidates for *in vivo* molecular imaging studies.
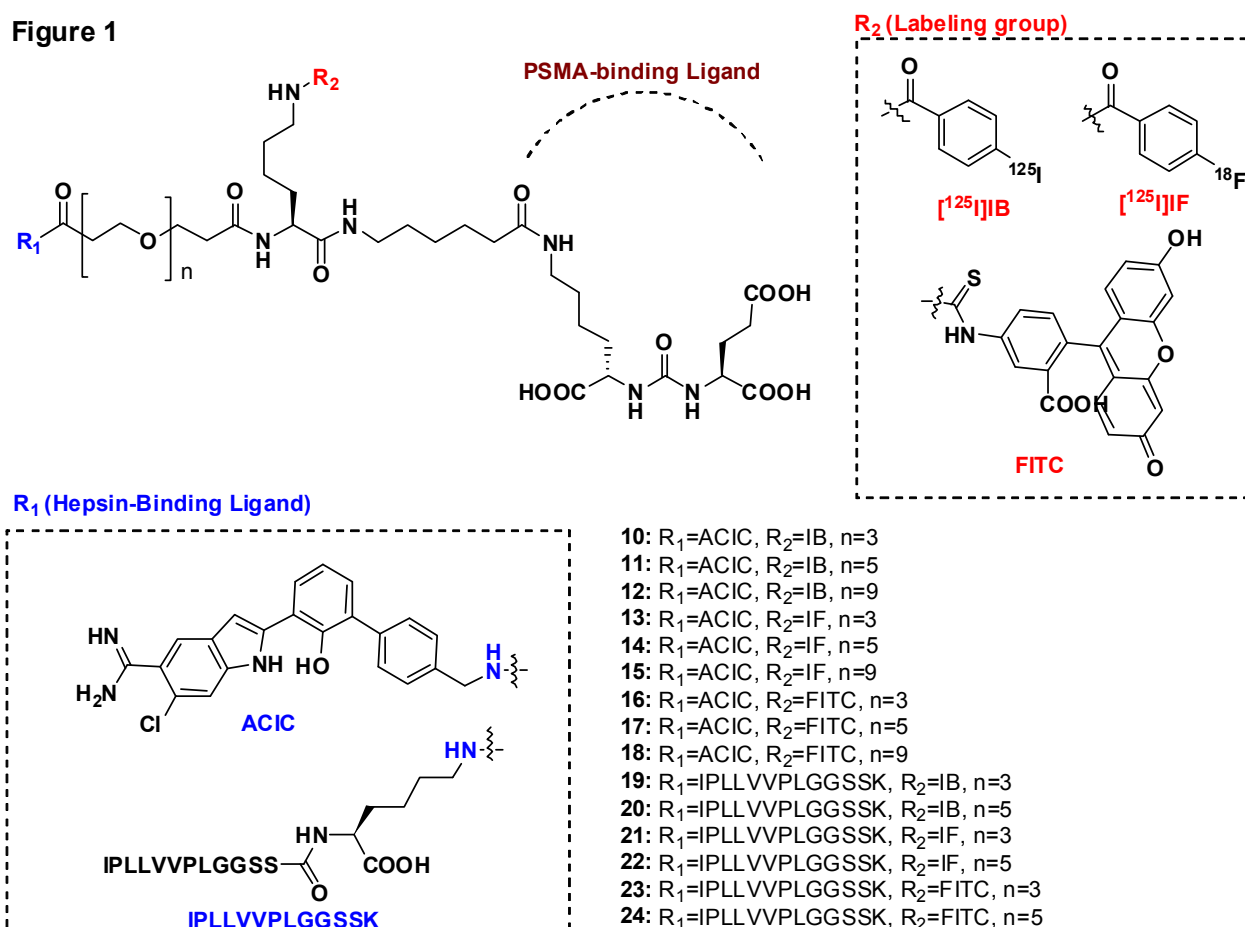
Valency is the number of separate connections that one microscopic entity makes with another. (15) In general, higher valency yields higher affinity. Bivalent ligands can be developed by attaching two pharmacophores with an optimal spacer. Our hypothesis of the original proposal was to discover novel heterobivalent conjugates to bind to PSMA and hepsin simultaneously. They can be further developed to be

more sensitive and potent PCa imaging agents. Due to the overexpression of PSMA and hepsin on the cell surface of metastatic PCa tumors, easy access of bivalent imaging probes to the target sites and high affinity for PCa-cells through heterobivalency can be possible.

B. Body

1. Specific Aims in Year 3

Figure 1

10: R_1=ACIC, R_2=IB, n=3
11: R_1=ACIC, R_2=IB, n=5
12: R_1=ACIC, R_2=IB, n=9
13: R_1=ACIC, R_2=IF, n=3
14: R_1=ACIC, R_2=IF, n=5
15: R_1=ACIC, R_2=IF, n=9
16: R_1=ACIC, R_2=FITC, n=3
17: R_1=ACIC, R_2=FITC, n=5
18: R_1=ACIC, R_2=FITC, n=9
19: R_1=IPLLVVPLGGSSK, R_2=IB, n=3
20: R_1=IPLLVVPLGGSSK, R_2=IB, n=5
21: R_1=IPLLVVPLGGSSK, R_2=IF, n=3
22: R_1=IPLLVVPLGGSSK, R_2=IF, n=5
23: R_1=IPLLVVPLGGSSK, R_2=FITC, n=3
24: R_1=IPLLVVPLGGSSK, R_2=FITC, n=5

We hypothesized that the sensitivity and accuracy of PCa diagnosis can be improved by dual-targeting of PSMA and hepsin. We proposed heterobivalent conjugates of PSMA/hepsin-binding ligands labeled with optical dyes, positron- and gamma-emitting radionuclides, in order to provide agents with enhanced affinity/avidity for PCa. The PSMA-binding ligand moiety of proposed conjugates is Lys-urea-Glu, which has been utilized as the key S1'-probing moiety for preparation of our PSMA imaging probes. We proposed three hepsin-binding ligands based on the small molecules reported as weak to moderate hepsin inhibitors. These are indole-5-carboximidamide scaffold (ACIC) crystallized with hepsin (14) and IPLLVVPL peptide obtained by phage-display microarrays. (15) The ultimate goals throughout the 3-yr project period was to synthesize the heterobivalent conjugates of the PSMA-ligand (Lys-urea-Glu) with the hepsin ligand (indole-5-carboximidamide) and to evaluate *in vitro* biological properties of IPLLVVPL peptide analogs for *in vivo* animal imaging studies with optical- or radionuclide-labeled conjugates in Final Year. The hetero-bivalent conjugates consist of three moieties that possess distinct roles for simultaneous targeting of PSMA and hepsin: (1) a high-affinity urea-based PSMA ligand, Lys-urea-Glu, (2) hepsin ligands derived from indole-5-carboximidamide or IPLLVVPL peptide, and (3) a nucleophilic functional group to be coupled with optical dyes.

2. Progress in Year 3

1. Aim 1: Synthesis and evaluation of heterobivalent conjugates of PSMA-ligand and ACIC-based hepsin ligand

Scheme 1. Synthesis of PSMA-hepsin conjugates
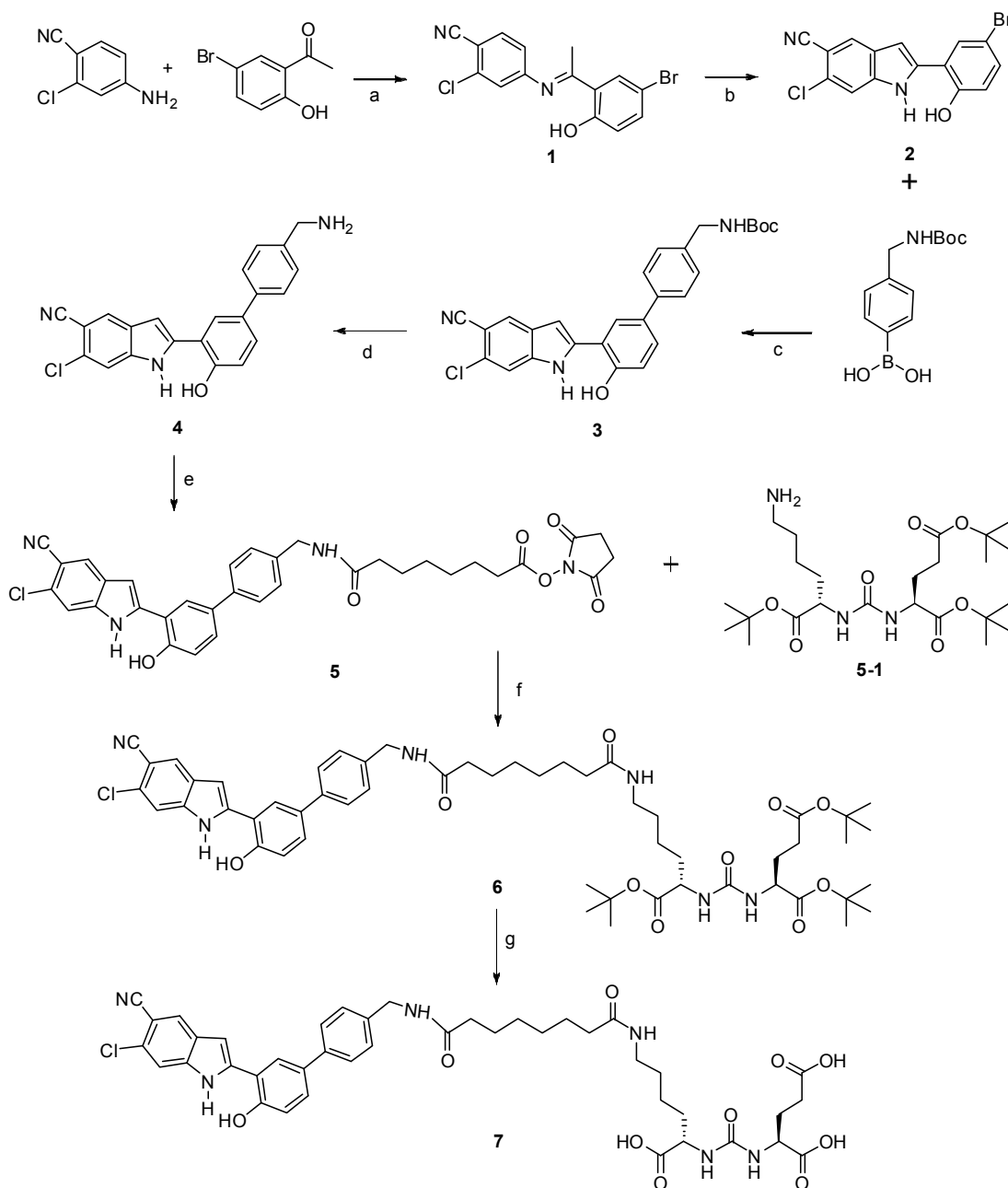
Reagents and conditions: (a) Piperidine, toluene, 120 °C, 25%; (b) Pd(OAc)$_2$, Bu$_4$NBr (2 eq), DMSO,100 °C, 30%; (c) Pd(PPh$_3$)$_4$, Cs$_2$CO$_3$, DMF/H$_2$O (5:1), 100 °C, 39%; (d) 25%TFA in DCM; (e) Et$_3$N, in DMF, 31%; (f) Et$_3$N, DMF, 21%; (g) 50%TFA in DCM, 43%

Scheme 1 outlines the synthesis of PSMA-hepsin conjugates with a suberic acid linker. Preparation of N-aryl imine **1** was achieved by reacting 4-amino-2-chlorobenzonitrile with 5-bromo-2-hydroxyacetophenone under a base-catalyzed condition (piperidine in toluene) at 120 °C in 20% yield. In order to obtain **1** in higher yield, a variety of reaction conditions were tested by changing the temperature, catalyst, and solvent. We tested toluene, *p*-xylene, DMF and ethanol as reaction solvent, piperidine, triethylamine, *p*-TsOH and NaHCO$_3$ as catalyst. The best yield was achieved when toluene as solvent and piperidine as catalyst were used at 120°C for 6 h using a Dean-Stark trap apparatus. The low yield in the imine formation step resulted from low nucleophilicity of amino group of

4-amino-2-chlorobenzonitrile because of the two electron-withdrawing groups, CN and Cl. When 4-amino-2-chlorobenzonitrile was replaced by aniline under the same reaction condition, the corresponding imine was obtained in 80 % yield.

The synthesis of indole analog **2** was achieved by applying the palladium-catalyzed oxidative cyclization reaction of N-aryl imine **1** by modifying the reported procedure. (16) The reaction can be operated under milder conditions and purification of the indole compounds could be handled efficiently. The major product **2**, 2-(5-bromo-2-hydroxyphenyl)-6-chloro-1H-indole-5-carbonitrile, was obtained in 30% yield with the regioisomer 2-(5-bromo-2-hydroxyphenyl)-4-chloro-1H-indole-5-carbonitrile as by-product in 5% yield. Two different reaction conditions were tested: (1) Pd(OAc)$_2$ (10 mol%), Bu$_4$NBr (2 eq), DMSO, 100 °C, 24 hr, and (2) Pd(OAc)$_2$ (10 mol%), Cu(OAc)$_2$ (3 eq), DMSO, 100 °C, 24 hr. The use of Bu$_4$NBr underwent the indole cyclization more effectively than Cu(OAc)$_2$ for the preparation of **2** with 2-fold increase in yield. The desired product **2** was separated easily from by-products using normal silica-gel chromatography. The low yield in the indole cyclization step resulted from the steric hindrance by hydroxyl group at the *ortho* position of 5-bromo-2-hdyroxyacetophenone.

The synthetic step for the preparation of indole-biphenyl analog **3** involved the use of palladium-catalyzed Suzuki cross-coupling reaction. The effects of solvent and base on the synthesis of **3** were studied using Pd(PPh$_3$)$_4$ as catalyst. DMF, ethanol, and DMSO were tested as solvent and Na$_2$CO$_3$, NaHCO$_3$ and Cs$_2$CO$_3$ as base. Among the tested conditions, the optimized condition was achieved by the use of Cs$_2$CO$_3$ as base and DMF/H$_2$O (5:1) as solvent at 100°C for 12 h. The amino group of 4-(aminomethyl)phenylboronic acid should be protected by *tert*-butyloxycarbonyl (Boc) group for the Suzuki cross-coupling reaction. Use of 4-(aminomethyl) phenylboronic acid have been plagued with coupling problems and did not afford **3**.

Removal of Boc group from compound **3** was achieved by the treatment of trifluoroacetic acid (TFA) in dichloromethane (DCM) to give the compound **4**. Compound **4** has nucleophilic functional group to react with the electrophilic moiety. The compound **4** was conjugated with suberic acid bis-(N-hydroxysuccinimide ester) under the basic condition to afford the N-hydroxysuccinimidyl suberate (DSS) ester intermediate **5** in 21% yield. The PSMA-hepsin conjugate **6** was prepared by reacting **5** with *tert*-butyl (^{t}Bu)-protected Lys-urea-Glu (**5-1**). The compound **5-1** was prepared from the commercial appropriately-protected lysine and glutamic acid in 3 steps in high yield by modifying the reported procedure. (10) The ^{t}Bu group of **6** was conveniently removed by using TFA at room temperature to give the PSMA-hepsin conjugate **7** in 43% yield. The chemical structure of **7** was fully confirmed by ^{1}H NMR and HRLC-MS ([M-H]$^-$: 829.2986) in negative mode as shown in Fig. 2.
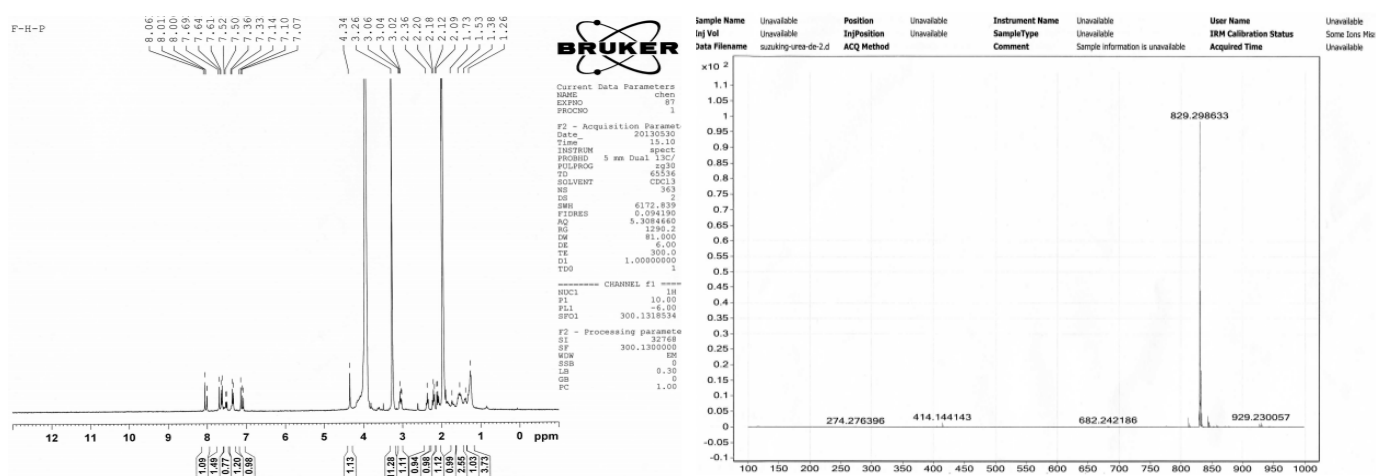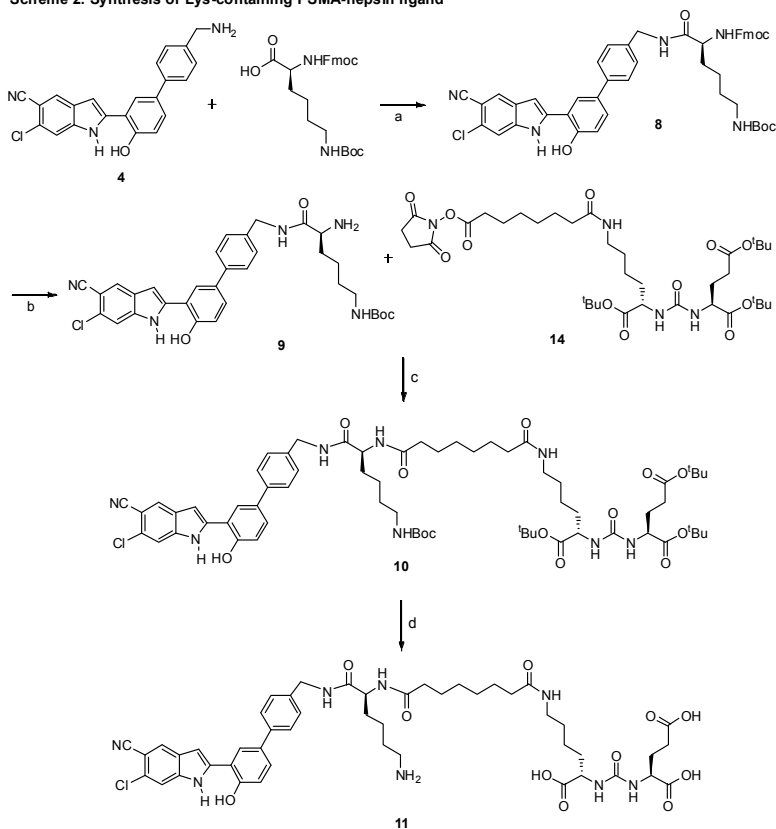

Fig. 2. ^{1}H NMR and HRMS data of compound 7

**Scheme 2. Synthesis of Lys-containing PSMA-hepsin ligand**

Reagents and conditions: (a) HATU (2eq), Et₃N (2eq), DMF, 50%; (b) 25% piperidine in DMF; (c) Et₃N, DMF 27% (2 steps) ; (d) 50%TFA in DCM, 40%

In order to prepare PSMA-hepsin conjugates labeled with optical dyes, a nucleophilic functional group was needed in compound **7**. Therefore, we synthesized lysine-linked PSMA-hepsin conjugate **11** as shown in Scheme 2. The primary amine moiety in **11** can react with a variety of electrophilic optical dyes as well as radiolabled prosthetic groups.

Reaction of indole-biphenyl amine **4** with commercial N_ε-Boc-N_α-Fmoc-(L)-lysine under the peptide-coupling conditions (HATU and TEA in DMF) afforded the compound **8** in 50% yield. Selective cleavage of Fmoc group of **8** was achieved by the treatment of 25% piperidine in DMF to give the compound **9.** Coupling of **9** with the compound **14,** which was obtained by reacting excess DSS ester with **5-1**, afforded the PSMA-hepsin conjugate **10** in 27% yield (2 steps). Removal of Boc and ᵗBu group of **10** using 50%TFA in dichloromethane (DCM) afforded the precursor **11** in 40% yield. Primary amine at the side chain of Lys is nucleophilic enough to react with the activated ester moiety of commercial Cy5.5 and Cy7.

Scheme 3. Synthesis of PSMA-hepsin conjugates labeled with optical dyes
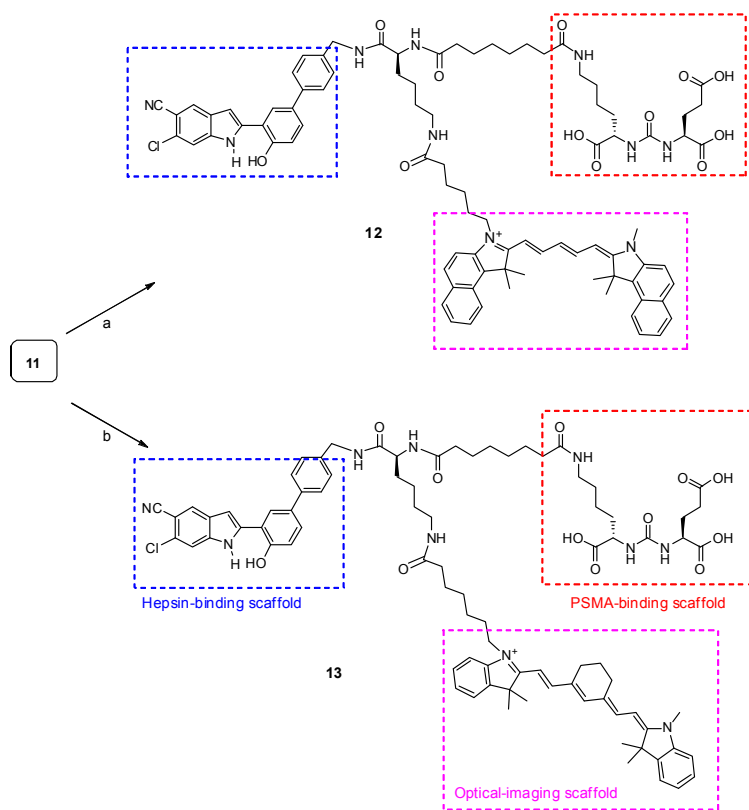
Reagents and conditions: (a) Tris-HCl (0.1M, pH=8.5), rt, 4h, 45%; (b) Tris-HCl (0.1M, pH=8.5), rt, 4 h, 36%

Scheme 3 outlines the synthetic route for PSMA/hepsin-targeted near-infrared (NIR) fluorescent agents. The commercially available amine-reactive active esters of Cy5.5 and Cy7 were conjugated with **11** in Tris HCl buffer (0.1 M, pH=8.5) at room temperature to afford dye-linked PSMA-hepsin conjugates **12** and **13** in moderate yield. These two final compounds could be purified by reversed-phase HPLC and analyzed by high resolution LC-MS. Purifications of compound **12** and **13** were achieved by HPLC with gradient method arrayed by 700 nm and 245 nm UV detectors. Retention time of **12** and **13** under the same gradient condition was 11.1 min and 9.0 min, respectively, indicating that the compound **13** is more hydrophilic than **12**. ESI-MS showed [M+H]²⁺ and [M+H]⁺ ion peaks of **12** and **13** in the positive modes as shown in Figure 3.
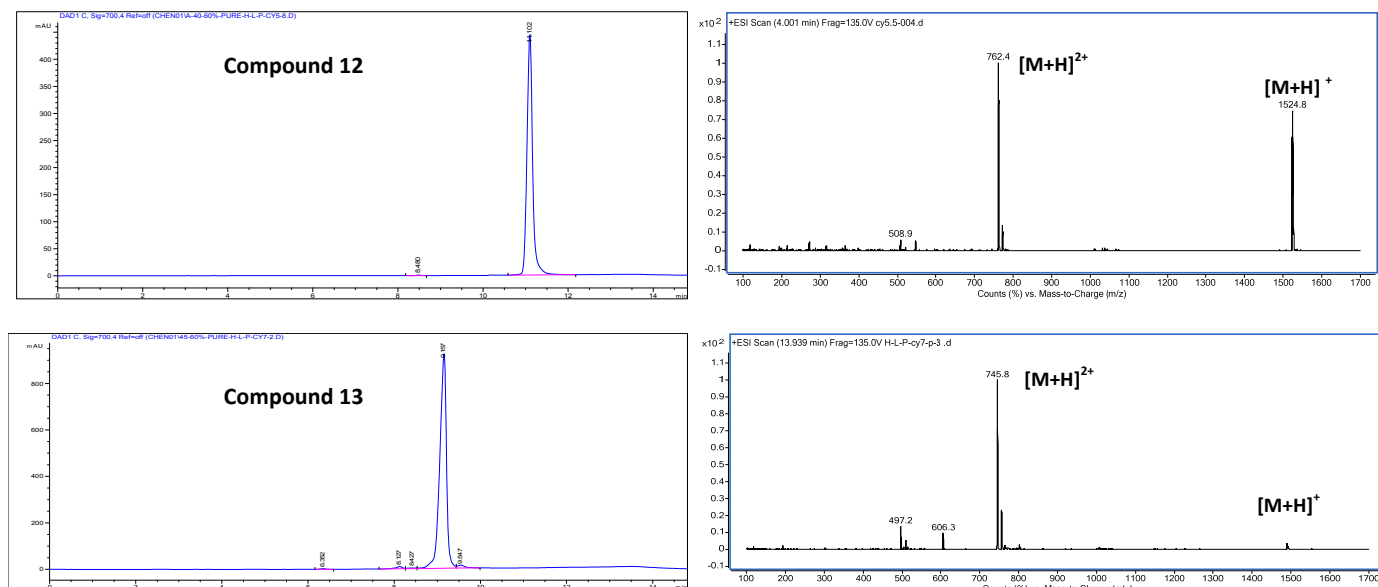
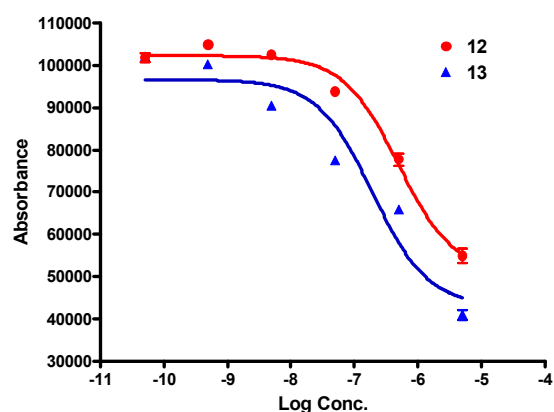**Fig. 3. HPLC chromatograms and HRMS spectra of compounds 12 and 13**

Fig. 4. IC$_{50}$ curve of compounds 12 and 13 using a fluorescence-based NAALADase assay

The IC$_{50}$ values of the conjugates **12-13** were measured using the using a fluorescence-based NAALADase assay. As shown in Figure 4, compounds **12** and **13** exhibited moderate inhibitory activities against PSMA *in vitro*. The K_i values of **12** and **13** were 99 nM and 39 nM, respectively. The results indicated that PSMA is well tolerated for the introduction of bulky groups at the P1 site of Lys-urea-Glu ligand if an optimal linker is attached.

2. **Aim 2:** *Synthesis and evaluation of heterobivalent conjugates of PSMA-ligand and peptide-derived hepsin ligand*
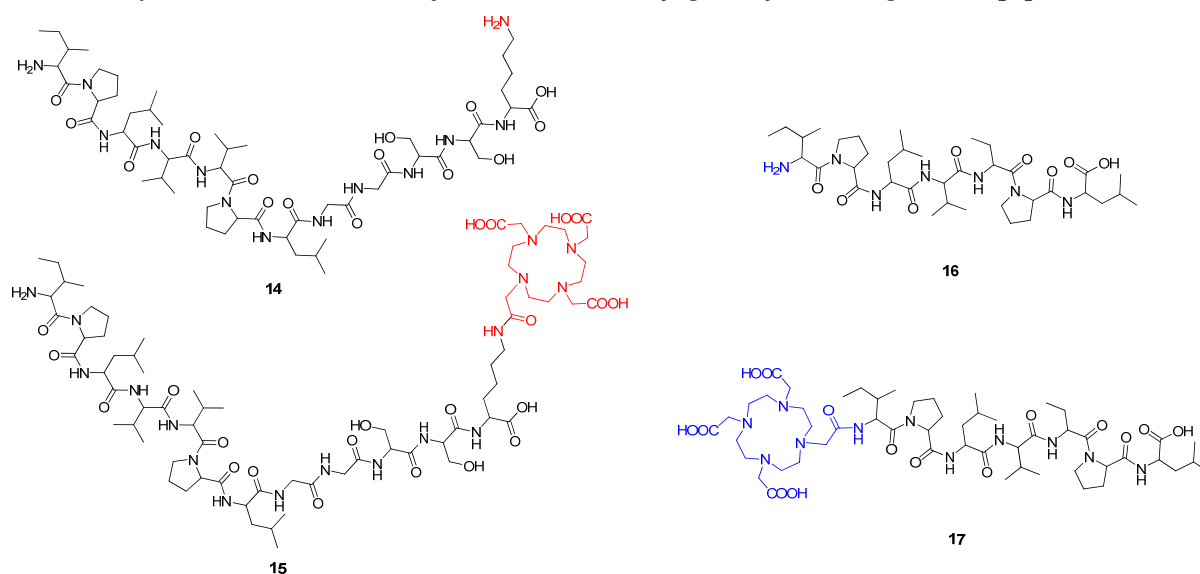

Fig.5. Chemical structures of DOTA-conjugated peptide analogs

Synthesis of conjugate of PSMA with IPLLVVPLGGSSK-peptide ligand: The IPLLVVPLGGSSK peptide, which has an affinity of 190 nM for hepsin-expressing PC3 cells (PC3/HPN cells), was identified by phage selection method by Kelly et al. (15). In Year 3, we attached the DOTA moieties to the IPLLVVPLGGSSK (**14**) peptide at the C-terminus and prepared the DOTA-conjugated IPLLVVPLGGSSK peptide **15** (Figure 5). Compound **16** (IPLLVVPL) was also conjugated with DOTA at the N-terminus to afford the compound **17** in moderate yield. The compounds **14-17** were purified by HPLC and used for *in vitro* hepsin inhibitory evaluation studies by following the vendor-provided procedure (www.rndsystems.com). The goal of comparing the binding pattern of both N-terminus and C-terminus derivatives was to determine the mode of binding of the IPLVVPL peptide to the hepsin protein and to find the suitable residues for introducing bulky optical dyes or radionuclide-carriers to the peptides. Docking prediction studies of compound **16** with the hepsin crystal structure using ZDOCK module (Discovery Studio 3.1, Accelrys Inc.) showed that the N-terminus of the IPLVVPL peptide projected towards the surface area of the hepsin active site as shown in Figure 6, indicating that the conjugation to the N-terminus of the peptides would be more favorable than C-terminus.
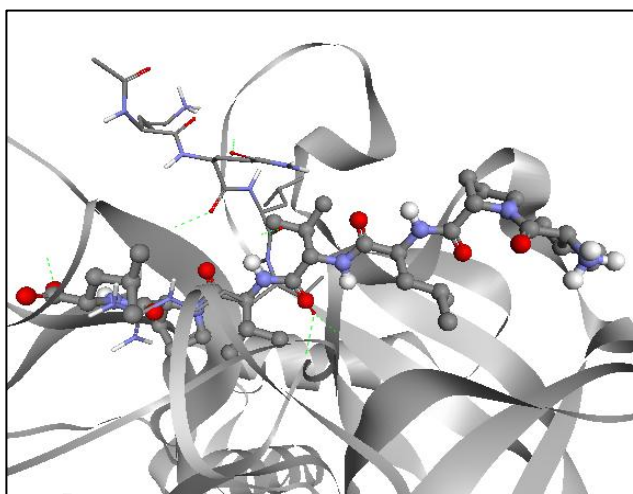

Fig.6. Binding mode of compound 16 to the active site of hepsin

In vitro hepsin binding affinities of the synthesized peptides were evaluated by the reported assay procedure (www.rndsystems.com). Initial hypothesis was that these analogs would bind to the hepsin active site and would accordingly inhibit the protease activity of hepsin on the known fluorogenic peptide substrate (Boc-Gln-Arg-Arg-AMC). The compounds for *in vitro* assay were prepared by serial dilution from 1 mM to 1 nM and were added to 0.01 μg of recombinant human hepsin (rh hepsin) with the fluorogenic substrate at 1 μM. After incubating the compounds with rh hepsin at 37 $^{\circ}$C for 1 hour, the fluorescence intensity for each compound was measured at excitation and emission wavelength of 380 nm and 460 nm, respectively. The obtained results exhibited that none of the 4 peptides (**14-17**) inhibit the protease activity of hepsin on its specific fluorogenic substrate. This indicated that these peptides might bind to a hepsin site that is different from the active site of the hepsin protein. In the original publication by Kelly's group (15), IPLVVPL peptide was discovered by a phage-display screen and its binding affinity for hepsin was evaluated with hepsin-overexpressing PC3 cells, not sole hepsin protein.

3. Aim 3: In vitro and In vivo imaging studies of heterobivalent radiolabeled agents

Development of PC3/ML cell lines expressing PSMA and hepsin (PC3/ML-PSMA-HPN):
In Year 3, we successfully developed the cell lines which express PSMA, hepsin, and PSMA/hepsin. The cell lines can be used for *in vitro* cell uptake studies as well as for generating xenograft mouse models for *in vivo* imaging studies. PC3/ML, a subline of PC3 cells with low expression of PSMA and hepsin, was obtained from Dr. Stearns' Lab, Drexel University School of Medicine.
PC3/ML-PSMA cell lines: Full length PSMA cDNA was cloned into pLV vector (lentiviral expression vector) and lentiviral particle were produced by transfecting HEK-293T cells with pLV-PSMA plasmids. PC3/ML cells were transfected with lentiviral particles and the single cell were isolated into 1 well of 96-well plate for clonal expansion using fluorescent activated cell sorter (FACS). Each clone was examined for PSMA expression *via* qRT-PCR and staining with YC-XI-46, the potent PSMA inhibitor conjugated with

the Cy5.5 dye. (17) PSMA-positive clones were selected for generating the cell line. The qRT-PCR result confirmed the PSMA gene expression from the PC3/ML-PSMA cells as shown in Figure 7.
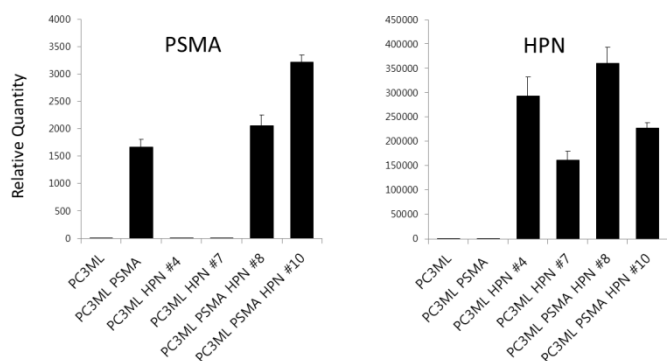

Fig. 7. qRT-PCR analyses for PSMA and HPN expression in the developed cell lines

PC3/ML-HPN cell lines: Full length HPN cDNA was cloned into pHIV-Luc vector (lentiviral expression vector for dual expression of fLUC and a gene of interest) and lentiviral particle were produced by transfecting HEK-293T cells with pHIV-Luc-HPN plasmids. PC3/ML cells were transfected with lentiviral particles and the single cell were isolated into 1 well of 96-well plate for clonal expansion using FACS. Each clone was examined for luciferase activity. Two best clones (clone #4 and #7) with luciferase activity were further examined for HPN expression by qRT-PCR.

The qRT-PCR result as shown in Figure 7 confirmed the *hepsin* gene expression of PC3/ML-HPN cell lines. The clone #4 was selected for FACS analysis and the cell uptake studies of compounds **12** and **13**.
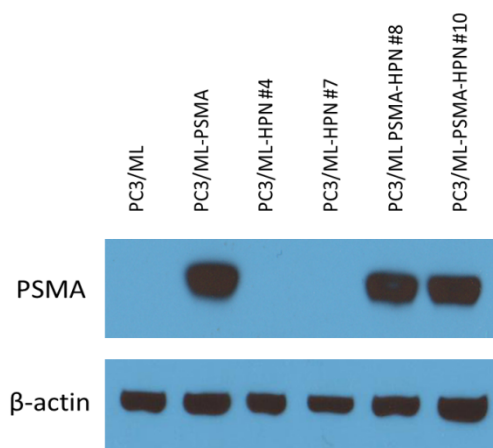

Fig. 8. qRT-PCR analyses for PSMA and HPN expression in the developed cell lines

PC3/ML-PSMA-HPN cell lines: PC3/ML-PSMA cell lines were transfected with lentiviral particles (pHIV-Luc-HPN) and the single cell were isolated into 1 well of 96-well plate for clonal expansion. The clones (#8 and #10) with luciferase activity were examined for HPN expression by qRT-PCR. PSMA expression of the clones was further confirmed by qRT-PCR, staining with YC-XI-46, and western blot analysis. The qRT-PCR result as shown in Figure 7 confirmed both PSMA and hepsin gene expression from the #8 and #10 clones of PC3/ML-PSMA-HPN cells. The 8th clone was selected for FACS analysis and the cell uptake studies of compounds **12** and **13**. Western blot analysis of the developed cell lines using the PSMA antibody exhibited the PSMA expression in PC3/ML-PSMA and PC3/ML-PSMA-HPN cell lines as shown in Figure 8. β-actin was used as an internal control for the western blot analysis.

The PSMA expression level of the 4 cell lines were evaluated using YC-XI-46 at 100 nM concentration.

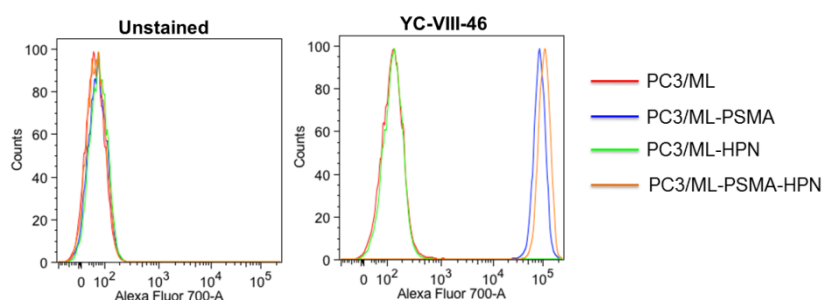

Fig. 9. FACS analysis of the cell lines with YC-XI-46 to confirm the PSMA expression

After staining cells with YC-XI-46, cells were analyzed using FACS (LSRII, BD biosciences). As shown in Figure 9, PC3/ML and PC3/ML-HPN cells remained negative with YC-XI-46 whereas PC3/ML-PSMA and PC3/ML-PSMA-HPN specifically stained with YC-XI-46, indicating the PSMA expression on these two cell lines.
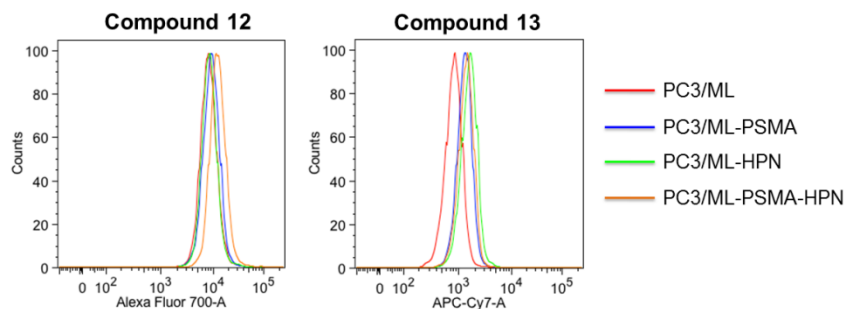
Fig. 10. Binding Analyses of compounds 12 and 13 by FACS labeling methods

Specific binding of the synthesized compounds **12** and **13** to the developed cell lines were evaluated at 100 nM concentration. As shown in Figure 10, compound **12** showed the non-specific binding to the PC3/ML cell as well as the three developed cell lines. The lipophilic aromatic rings of the hepsin-binding scaffold in **12** increases the overall hydrophobicity of **12**, thus, resulting in the binding to the cell membranes nonspecifically. However, compound **13** with Cy 7 is less lipophilic than **12** and exhibited a slightly stronger specific-binding for the PC3/ML-PSMA, PC3/ML-HPN, and PC3/ML-PSMA-HPN as compared to PC3/ML (see the right panel in Figure 10).

C. Key Research Accomplishments:

✓ We established an efficient synthetic strategy to prepare PSMA-hepsin conjugates for structure-activity relationship (SAR) studies. Using the synthetic procedure, we successfully synthesized two novel PSMA-hepsin conjugates labeled with optical dyes (Cy5.5 and Cy7) and evaluated *in vitro* biological activities.

✓ We successfully developed the PC3-derived cell lines which express PSMA, hepsin, and PSMA/hepsin. PSMA and hepsin expression on the three cell lines was confirmed by qRT-PCR, FACS, and Western blot analysis. These cell lines were utilized for *in vitro* cell uptake studies and will be used for generating xenograft mouse models for *in vivo* imaging studies.

D. Reportable Outcomes: None

E. Conclusion: In Year 3 we were able to prepare two PSMA-hepsin conjugates labeled with optical dyes such as Cy5.5 and Cy7. They were obtained by conjugating the PSMA-binding ligand (Lys-urea-Glu) with a simplified moiety of indole-5-carboximidamide scaffold with a suberic acid linker. Although they exhibited moderate binding affinity for PSMA, no significant selective uptake of PSMA- and PSMA/hepsin-expressing cell lines was observed. This was due to the high lipophilicity of the conjugates and the close proximity between the PSMA-binding scaffold and the hepsin-binding scaffold of the conjugates. We also synthesized two DOTA-linked IPLLVVPLGGSSK-peptides and evaluated their inhibitory activities against hepsin protease. None of them exhibited a meaningful inhibition against hepsin protease. Regarding the biology aspect, we successfully developed the cell lines which express PSMA, hepsin, and PSMA/hepsin from PC3/ML cells for the *in vitro* cell uptake studies and the *in vivo* imaging studies in Final Year.

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